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13. ABSTRACT *(Maximum 200 Words)*

This research distinguishes mechanisms through which activated Ras initiates rat mammary gland carcinogenesis. Experiments expressing H-Ras and K-Ras chimeric proteins in mammary gland (Aim 1A) investigate hypothesis one: differential rat mammary gland carcinogenesis results from differences in the last 20 amino acids of H-Ras and K-Ras. K-Ras with an H-Ras c-terminus seems as tumorgenic as H-Ras, supporting hypothesis one: however, H-Ras with an K-Ras c-terminus also seems as tumorgenic as H-Ras, suggesting a unique characteristic of intact K-Ras reduces mammary carcinogenesis.

Expression of Raf-Caax (Aim 1B), does not cause tumors, suggesting hypothesis two: no individual Ras effector will initiate transformation, rather multiple effectors must synergise. Expressing Ras effector loop mutants (ELM), (Aim 2A), weakens hypothesis two. Each Ras ELM (G37 activates RalGDS, E38 activates Raf, and C40 activates PI3K), individually causes tumors. E38-Ras, which activates Raf, is tumorgenic yet Raf-Caax is not. These results suggested E38 Ras is activating an additional effector pathway(s), or that Raf-Caax is defective for *in situ* tumor formation. Early result tumor formation from a-terminus truncated Raf (ΔRaf) shows Raf-Caax is defective, and eliminates hypothesis two.

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FOREWORD

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D. M. Tull 9-13-00

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Introduction:

This research distinguishes mechanisms through which activated Ras induces rat mammary gland carcinogenesis. Aim 1 focuses on differences between H-Ras and K-Ras, while Aim 2 focuses on different Ras effector pathways. In situ expression of activated H-Ras is 5-10 times more tumorgenic than K-Ras in rat mammary gland. Experiments expressing H-Ras and K-Ras chimera proteins in rat mammary glands are distinguishing critical differences between H-Ras and K-Ras proteins. Activated Ras proteins bind other signal transduction proteins, such as Raf, PI3K, and RalGDS. We don't know which Ras effector proteins are necessary and/or sufficient for initiation of mammary tumors. *In situ* expression experiments with Ras effector loop mutants (ELM's), and Raf-Ras fusion proteins (Raf-H-Caax, Raf-K-Caax), are distinguishing critical Ras effector proteins. These results have important implications for targeted treatment of different tumor types with novel chemotheraputic agents.

Body:

Aim 1 is to distinguish which H-Ras and K-Ras protein domains result in different potential to transform *in situ* mammary epithelial cells. The hypothesis of Aim 1 is; the different potential of H-Ras and K-Ras to initiate rat mammary gland carcinogenesis, results from differences in the last 20 amino acids of H-Ras and K-Ras. This hypothesis is being tested with expression of chimeric Ras proteins.

Aim 1A is comparing differences in rat mammary tumor formation resulting from expression of activated H-Ras and K-Ras with their carboxyl ends exchanged. Expressing H-Ras and K-Ras chimeric proteins in rat mammary gland partially supports the hypothesis of Aim 1. K-Ras with an H-Ras c-terminus seems as tumorgenic as H-Ras, supporting the hypothesis of Aim 1: however, H-Ras with an K-Ras c-terminus also seems as tumorgenic as H-Ras, suggesting a unique characteristic of intact K-Ras is less tumorgenic in mammary gland.

Aim 1B was to compare differences in rat mammary tumor formation resulting from expression of Raf activated by fusion to the carboxyl end of H-Ras (Raf-H-Caax) to Raf activated by fusion to the carboxyl end of K-Ras (Raf-K-Caax). The results of Aim 1B don't reflect on the hypothesis of Aim 1, since neither form of Raf resulted in a single tumor. The results of Aim 1B suggest the hypothesis of Aim 2: no individual Ras effector will initiate transformation, multiple effectors must synergise.

New antibodies and fractionation techniques that allow staining for individual Ras family members and fractionation of plasma membrane micro domains make the methods proposed for Aim 1C antiquated. These newer methods will be employed if follow-up Aim 1A experiments validate the hypotheses of Aim 1.

Aim 2 is to distinguish the pathway(s) critical to transformation of *in situ* mammary epithelial cells by activated H-Ras. As mentioned earlier, the results of Aim 1B suggest the hypothesis of Aim 2: no individual Ras effector will initiate transformation, multiple effectors must synergise. Raf is thought to be the most tumorgenic of Ras effectors, so lack of tumor formation from expression of Raf-Caax, suggests Raf must synergise with an additional Ras effector(s). The results from Aim 1B contrast with those from Aim 2A.

Aim 2A was expressing individual Ras effector loop mutants (ELM's), which target particular Ras effectors (G37-Ras activates RalGDS, E38-Ras activates Raf, and C40-Ras activates PI3K). The results of Aim 2A experiments substantially weaken the hypothesis of Aim 2. Each of the Ras ELM's causes tumors individually. Tumors from expressing G37 or C40 Ras had a long latency (12-16 weeks), similar latency to tumors from expression of non-activated wt-H-Ras. E38-Ras, which activates Raf, is very tumorgenic with a short latency (3-5 weeks), as seen with activated H-Ras. This suggests Raf is the critical tumor generating effector of activated H-Ras. The coexpression experiments proposed for Aims 2B and 2C are no longer relevant due to the results of Aim 2A.

The contrasting results from Aim 1B and Aim 2A suggest Raf-Caax is defective for *in situ* tumor formation, or (and) E38-Ras is activating an additional effector pathway(s). RT-PCR of mRNA from Raf-H-Caax or Raf-K-Caax infused glands, shows some cells in the gland are still expressing the RNA months after infusion. Transformation of cultured cells by the Raf-Caax vectors, and immunostaining of infected cells has confirmed expression of the Raf proteins from the vectors. The first three Raf proteins expressed carried epitope tags for future identification (tag-wt-Raf, tag-Raf-H-Caax, and tag-Raf-K-Caax). To rule out an immunoresponse to the protein tag, Raf expression vectors were reconstructed without tags (wt-Raf, Raf-H-Caax, and Raf-K-Caax). A-terminus truncation of Raf is another way to increase its kinase activity. To further investigate the carcinogenic potential of Raf, a-terminus truncations were also constructed (ΔRaf, ΔRaf-H-Caax, and ΔRaf-K-Caax). Of the nine Raf vectors tested, only ΔRaf causes tumors.

Formation of rat mammary tumors from expression of Ras-ELM's weakened hypothesis two, and tumor formation from ΔRaf eliminates hypothesis two. Tumor formation from ΔRaf also shows Raf-Caax is defective for *in situ* tumor formation. Furthermore, tumor formation from ΔRaf but not from ΔRaf-Caax suggests membrane targeting of activated Raf can actually block tumorgenesis. This is very preliminary ΔRaf data: further infusions are necessary to substantiate these findings.

Key research accomplishments:

- Constructed, concentrated, titered, and tested for absence of helper virus in JR-H-Ras-H-Caax, JR-H-Ras-K-Caax, JR-K-Ras-K-Caax, and JR-K-Ras-H-Caax retroviral vectors (domain swap vectors).

- Infused glands with domain swap vectors and palpated; there were 26 rats per group.
  --Suggests unique characteristic of intact K-Ras is less transforming. Both chimeras are strongly tumorgenic like H-Ras.

- Constructed, concentrated, titered, and tested for absence of helper virus in JR-tag-Raf-H-Caax, JR-tag-Raf-K-Caax, and JR-tag-wt-Raf retroviral vectors (tag-Raf-Caax vectors).

- Infused glands with tag-Raf-Caax vectors and palpated; there were 12 rats per group.
  --Suggests Raf alone is not able to transform mammary epithelial cells *in situ*.

- RT-PCR on RNA from tag-Raf-Caax infused glands.
  --Confirmed expression of tag-Raf-Caax RNA's in infused mammary glands.

- Immunostaining of tag-Raf-Caax infected cultured cells.
  --Confirmed expression of Raf-Caax proteins in infected cultured cells.

- Constructed, concentrated, titered, and tested for absence of helper virus in JR-V12-H-Ras, JR-G37-V12-H-Ras, JR-E38-V12-H-Ras, and JR-C40-V12-H-Ras retroviral vectors (Ras-ELM vectors).

- Infused glands with Ras-ELM vectors and palpated; there were 12 rats per group.
  --Suggest Ras can generate tumor formation by activating any individual effector.
  --Suggest Raf activated by Ras (without PI3K or RalGDS) transforms like activated Ras (fast), while PI3K or RalGDS activated by Ras transform like non-activated wt-H-Ras (slow).

- Infused glands with JR-V12-H-Ras or JR-E38-V12-H-Ras ELM vector and palpated, there were 12 rats per group.
  --Confirmed Raf activated by Ras is similar to transformation from activated Ras.
  --This suggest Raf is the critical effector of Ras in mammary tumorgenesis.

- Infused glands with Ras-ELM or tag-Raf-Caax vectors and palpated; there were 12 rats per group.
  --Confirmed Raf activated by Ras (without PI3K or RalGDS) transforms like activated Ras, while tag-Raf-Caax fails to transform.
  --Confirmed PI3K or RalGDS activated by Ras, results in much greater latency to tumor formation, than Raf activated by Ras, or activated Ras.

- Constructed, concentrated, titered, and tested for absence of helper virus in JR-Raf-H-Caax, JR-Raf-K-Caax, JR-wt-Raf, JR-ΔRaf-H-Caax, JR-ΔRaf-K-Caax, and JR-ΔRaf retroviral vectors (Raf-Caax, and ΔRaf vectors).

- Infused glands with Raf-Caax, and ΔRaf vectors and palpated; there were only 3 rats per group.
  --Shows ΔRaf is tumorgenic in rat mammary gland.
  --Suggest Raf-Caax is defective for tumorgenesis.
  --Suggest ΔRaf-Caax is also defective for tumorgenesis
  --This suggest membrane targeting of activated Raf inhibits tumorgenesis.

Reportable Outcomes:

McFarlin, D.R., Kennan, W.S. and Gould M.N., "Ras signaling through Raf is more tumorgenic in rat mammary gland than Ras signaling through PI3K or RalGDS."; Abstract #2475 in Proceedings of the American Association for Cancer Research, vol.40, p.374, 1999.

This was presented as a poster at the 90th annual meeting of the American Association for Cancer Research in Philadelphia, PA in April of '99.

A similar poster presentation was given at the "Genetics, Genomes, and Molecules" symposium on the UW-Madison campus in May of '99

Copy of Reportable Outcome:

Ras signaling through raf is more tumorgenic in rat mammary gland than ras signaling through PI3K or RalGDS. McFarlin, D.R., Kennan, W.S. and Gould, M.N. *McArdle Laboratories for Cancer Research, University of Wisconsin, Madison, WI 53792*

Ras proteins are guanine nucleotide binding proteins localized at the plasma membrane. In its active state, the effector loop of Ras binds many other signal transduction proteins including Raf, PI3K, and RalGDS. Ras with a valine substitution for glycine at amino acid 12 (V12-Ras) is continually active and oncogenic. Ductal infusion of rat mammary glands, with retroviral vectors conferring expression of V12-Ras, results in tumor formation from infected mammary epithelial cells (MEC). V12-Ras which also has a glutamic acid substitution in the effector loop at amino acid 38 (E38-V12-Ras) is not able to bind PI3K or RalGDS but is still continually active for binding Raf. Expression of E38-V12-Ras in rat mammary gland generates an equivalent number of tumors as expression of V12-Ras. C40-V12-Ras is not able to bind Raf or RalGDS but is still binds PI3K. Expression of C40-V12-Ras in rat mammary gland generates significantly fewer tumors than V12-Ras, or E38-V12-Ras. G37-V12-Ras is not able to bind Raf or PI3K but is still binds RalGDS. Expression of G37-V12-Ras in rat mammary gland generates a similar number of tumors as C40-V12-Ras and significantly fewer tumors than V12-Ras, or E38-V12-Ras. Equivalent tumor numbers resulting from expression of V12-Ras and E38-V12-Ras, substantiates Raf as a considerable contributor to transformation by Ras. Generation of significantly fewer tumors by expression of C40-V12-Ras and G37-V12-Ras suggests interaction of PI3K and RalGDS contribute less to MEC transformation by Ras than interaction with Raf.